2017

Immunohistochemistry Protocols

EXPERIMENTAL PATHOLOGY LABORATORY, WIMR I, RM. 4018

Protocol Index

Rabbit Anti-Human Androgen Receptor (Cell Signaling XP® Rabbit mAb, D6F11, 5153P) Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue

1. Five µm thick sections were cut from FFPE human tissue and mounted on charged slides.
2. Slides were placed in an 80 °C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes ×3) and hydrated through graded alcohols to deionized water.
4. HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10 mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
8. Serum block using 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at 1:400 overnight at 4º C.
11. PBS rinse ×3.
12. Incubate with Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
13. PBS rinse ×3.
14. Stain with DAB (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. dH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse in tap H_2O 10 minutes.
18. dH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Rabbit Anti-Human Polyclonal Androgen Receptor (AR (N20) Santa Cruz Biotechnology, Inc., sc-816) Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue

1. Five µm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Slides were placed in an 80 °C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes ×3) and hydrated through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10 mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at 1:1600 overnight at 4º C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly, MA) 30 minutes at room temperature.
13. PBS rinse ×3.
14. Stain with DAB (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse in tap H_2O for 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Anti-CD1α Rabbit Polyclonal (Abcam, ab136922) Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue

1. Five µm thick sections were cut from FFPE human tissue and mounted on charged slides.
2. Slides were placed in an 80 °C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes ×3) and hydrated through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10 mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum at 1:200 overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly, MA) 30 minutes at room temperature.
13. PBS rinse ×3.
14. Stain with DAB (Cell Signaling Technology, Beverly, MA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) 1 minute.
17. Rinse in tap H_2O for 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Rat Anti-Mouse CD8a (ebioscience, 14-0808, Clone: 4SM15) Immunohistochemistry on FFPE Mouse Tissue

1. Slides were cut at 5 μm, mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80 °C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH_2O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20) for three minutes in Biocare Decloaker.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes
8. Serum block for 60 minutes at room temperature. Use 10% goat serum in PBS.
9. Incubate with primary antibody at 1:800 in PBS with 1% goat serum overnight at 4ºC.
10. Rinse for 5 minutes in PBS ×3.
11. Anti-rat IgG (HRP, Rat, Vector, MP-7444) 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS ×3.
13. Stain with DAB (Cell Signaling, #8059S) 1 minute at room temperature.
14. Rinse in dH_2O.
15. Counterstain 1 minute with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H_2O for 10 minutes.
17. Rinse in dH_2O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

Rabbit mAb Anti-CD14(abcam ab133335, EPR3653) Indirect Immunofluorescence on Formalin Fixed Paraffin Embedded Human Tissue

1. Tissue was cut at 5 µm, mounted on charged slides, then incubated in an 80° °C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 minutes in dH_2O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM citric acid, 0.05% Tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 minutes.
7. Rinse in PBS.
8. Serum block for one hour at room temperature. Use 10% goat serum in PBS.
9. Incubate with primary antibody at 1:1000 in PBS with 1% goat serum and 0.1% Triton X-100.
10. Rinse for 5 minutes in PBS ×3.
11. Incubate with Alexa Fluor 647 goat anti-rabbit IgG H&L (Invitrogen) in PBS at 1:500 for one hour at room temperature in the dark.
12. Rinse for 5 minutes in PBS ×3.
13. Rinse in dH_2O 5 minutes
14. Cover slip with Prolong Gold antifade reagent with DAPI (Invitrogen).

Anti-CD31 Rabbit Polyclonal (Abcam, ab28364) Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue

1. Five µm thick sections were cut from FFPE murine tissue and mounted on charged slides.
2. Place slides in an 80 °C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes ×3) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10 mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum, Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at 1:400 overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
13. PBS rinse ×3.
14. Stain with DAB substrate (Cell Signaling Technology, Beverly, MA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse in tap H_2O for 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Rat Anti-mouse CD45 (BD Pharmingen, 553076) Immunohistochemistry on FFPE Mouse Tissue

1. Deparaffinize and hydrate sections.
2. Rinse for 5 minutes in dH_2O.
3. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20), three minutes in Biocare Decloaker.
4. Cool slides for 30 minutes.
5. Block endogenous peroxidase with 3% H_2O_2 in PBS for 20 minutes.
6. Serum block for 30 minutes with 10% goat serum in PBS.
7. Incubate with primary antibody at 1:6400 in PBS with 1% goat serum overnight at 4ºC.
8. Rinse for 5 minutes in PBS ×3.
9. Incubate with anti-rat IgG (Vector, MP-7444) for 30 minutes at room temperature.
10. Rinse for 5 minutes in PBS ×3.
11. Incubate with Signal Stain® DAB (Cell Signaling Technology, 8059S) for 1 minute at room temperature.
12. Rinse in dH_2O.
13. Counterstain for 1 min with Mayer's hematoxylin (Sigma-Aldrich, MHS32).
14. Rinse in tap H_2O for 10 minutes
15. Dehydrate through graded alcohols to xylene.
16. Coverslip with Permount.

Mouse Anti-Rat CD 163 Immunohistochemistry on FFPE Sections with Polymeric Secondary

1. Tissue was cut at 5 µm, mounted on charged slides, then incubated in an 80̊ C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 minutes in dH_2O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM citric acid, 0.05% Tween 20), or Tris-EDTA, pH 9.0(10mM tris base, 1 mM EDTA, 0.05% Tween 20) for 3 minutes in Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 minutes.
7. Rinse in PBS.
8. Block endogenous peroxidase with 3% H_2O_2 in PBS for 20 minutes.
9. Serum block for one hour at room temperature using 10% goat serum in PBS.
10. Avidin block (0.001% avidin in PBS) for 10 min at room temperature.
11. Biotin block (0.001% biotin in PBS) for 10 min at room temperature.
12. Incubate with primary antibody diluted in PBS with 1% goat serum and 0.01% Triton X-100 overnight at 4ºC.
13. Rinse for 5 minutes in PBS ×3.
14. Incubate with SignalStain Boost IHC Detection Reagent, HRP,mouse (Cell Signaling Technologies, Danvers,MA) for 30 minutes at room temperature.
15. Rinse for 5 minutes in PBS ×3.
16. Stain with DAB (Vector Laboratories, Burlingame, CA).
17. Rinse in tap water.
18. Counterstain 1 min with Mayer's hematoxylin.
19. Rinse in tap H_2O fo 10 minutes.
20. Rinse in dH_2O for 5 minutes.
21. Dehydrate through graded ethanols to xylene.
22. Cover slip with Permount.

Rabbit Anti-Human Cleaved Caspase 3(Cell Signaling, 9661S) Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue

1. Tissue was cut at 5 µm, mounted on charged slides, then incubated in an 80̊ C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in 3 changes of xylene, 5 minutes each.
3. Hydrate sections through graded ethanols to deionized water.
4. Rinse for 5 minutes in dH_2O.
5. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM citric acid, 0.05% Tween 20), for 3 minutes in a Biocare decloaker (Biocare Medical, Concord, CA).
6. Cool slides for 30 minutes.
7. Rinse in PBS.
8. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes.
9. Serum block for one hour at room temperature using 10% goat serum in PBS.
10. Incubate with primary antibody at 1:100 in PBS with 1% goat serum and 0.01% Triton X-100 overnight at 4ºC.
11. Rinse for 5 minutes in PBS ×3.
12. Incubate with Signal Stain® Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling, 8114S) for 30 minutes at room temperature.
13. Rinse for 5 minutes in PBS ×3.
14. Stain with DAB (Cell Signaling, 8059S) for 1 minute at room temperature.
15. Rinse in tap water.
16. Counterstain for 1 minute with Mayer's hematoxylin (Sigma, MHS32).
17. Rinse in tap H_2O for 10 minutes.
18. Rinse in dH_2O for 5 minutes.
19. Dehydrate through graded ethanols to xylene.
20. Cover slip with Permount.

Anti-Collagen VII Rabbit Polyclonal (Abcam, ab93350) Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue

1. Five µm thick sections were cut from FFPE human tissue and mounted on charged slides.
2. Slides were placed in an 80 °C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes ×3) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) Proteinase K (Dako, S3020) for 10 minutes at room temperature.
5. PBS rinse.
6. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
7. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
8. PBS rinse.
9. Incubate with primary antibody in PBS with 1% goat serum at 1:1600 overnight at 4 °C.
10. PBS rinse ×3.
11. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
12. PBS rinse ×3.
13. Stain with DAB (Cell Signaling Technology, Beverly, MA) for 1 minute.
14. DH_2O rinse.
15. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) 1 minute.
16. Rinse in tap H_2O for10 minutes.
17. DH_2O rinse.
18. Dehydrate through graded alcohols to xylene.
19. Cover slip with Permount.

Rabbit Anti- Cox2 (Cell Signaling, #12282) Immunohistochemistry on FFPE Human Tissue

1. Slides were cut at 5 μm, mounted on charged slides, and dried at room temperature overnight.
2. Place lides in 80 °C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH_2O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature using 10% goat serum in PBS.
9. Incubate with primary antibody at 1:200 in PBS with 1% goat serum overnight at 4ºC.
10. Rinse for 5 minutes in PBS ×3.
11. Incubate with Signal Stain® Boost IHC Reagent (HRP, Rabbit, Cell Signaling #8114S) for 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS ×3.
13. Stain with DAB (Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH_2O.
15. Counterstain for 1 minute with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H_2O 10 minutes.
17. Rinse in dH_2O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

Anti-CREB (Cell Signaling, #9197 Rabbit mAb (48H2)) Immunohistochemistry on FFPE Mouse Tissue

1. Slides were cut at 5 μm, mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80˚C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH_2O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 minutes
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature using 10% goat serum in PBS.
9. Incubate with primary antibody at 1:6400 in PBS with 1% goat serum overnight at 4ºC.
10. Rinse for 5 minutes in PBS ×3.
11. Incubate with Signal Stain® Boost IHC Reagent (HRP, Rabbit, Cell Signaling #8114S) for 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS ×3.
13. Stain with DAB (Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH_2O.
15. Counterstain for 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H_2O 10 minutes.
17. Rinse in dH_2O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

Chemokine (C-X-C motif) Receptor 4 Goat Anti-mouse Polyclonal Indirect Immunohistofluorescence on FFPE Mouse Tissue

1. Formalin-fixed paraffin-embedded tissue was cut at 5 µm, mounted on charged slides, then placed in an 80˚C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in three changes of xylene, five minutes each.
3. Hydrate slides through graded ethanols to deionized water.
4. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mMcitric acid, 0.05% Tween 20), for 3 minutes in a Biocare Decloaker (Biocare Medical, Concord, CA). Let slides cool to room temperature.
5. Serum block for 1 hour at room temperature. Use 10% rabbit serum in PBS.
6. Incubate with primary antibody diluted to 1:50 in PBS with 1% rabbit serum overnight at 4˚C.
7. Rinse for 5 minutes in PBS ×3.
8. Incubate with Rabbit anti-goat Alexa Fluor 488 (H+L) (Life Technologies, Grand Island, NY) at 1:500 in PBS for 30 minutes at room temperature in the dark.
9. Rinse for 5 minutes in PBS ×3.
10. Rinse in deionized water ×3.
11. Cover slip with ProLong Gold antifade reagent with DAPI (Life Technologies, Grand Island, NY).

Positive Control Tissue – Mouse Spleen

Monoclonal Rabbit Anti-E-cadherin(24E10) (Cell Signaling, 3195S) Indirect Immunohistofluorescence on FFPE Human Tissue

1. Formalin-fixed paraffin-embedded tissue was cut at 5 µm, mounted on charged slides, then placed in an 80˚C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in three changes of xylene, five minutes each.
3. Hydrate slides through graded ethanols to deionized water.
4. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM citric acid, 0.05% Tween 20), for 3 minutes in a Biocare Decloaker (Biocare Medical, Concord, CA). Let slides cool to room temperature.
5. Serum block for 1 hour at room temperature using 10% goat serum in PBS.
6. Incubate with primary antibody diluted to 1:200 in PBS with 1% goat serum overnight at 4˚C.
7. Rinse for 5 minutes in PBS ×3.
8. Incubate with goat anti-rabbit Alexa Fluor 488 (H&L) at 1:500 in PBS 30 minutes at room temperature in the dark.
9. Rinse for 5 minutes in PBS ×3.
10. Rinse in deionized water ×3.
11. Cover slip with Crystal Mount (Biomeda, M03).

Rabbit Anti-human eNOS (Thermo, PA1-037) Indirect Immunohistochemistry on FFPE Mouse Tissue

1. Formalin-fixed paraffin-embedded tissue was cut at 5 µm, mounted on charged slides, then placed in an 80 °C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in three changes of xylene, five minutes each.
3. Hydrate slides through graded ethanols to deionized water.
4. Perform antigen retrieval in Tris-EDTA buffer, pH 9.0 (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0), for 3 minutes in a Biocare Decloaker (Biocare Medical, Concord, CA). Let slides cool to room temperature.
5. PBS rinse.
6. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
7. Serum block for 1 hour at room temperature using 10% goat serum in PBS.
8. Incubate with primary antibody diluted to 1:100 in PBS with 1% goat serum and 0.01% triton X-100 overnight at 4˚C.
9. Rinse for 5 minutes in PBS ×3.
10. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit)(Cell Signaling Technology, Beverly,MA) for 30 minutes at room temperature.
11. Rinse for 5 minutes in PBS ×3.
12. Stain with DAB (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
13. dH_2O rinse.
14. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
15. Rinse with tap H_2O for 10 minutes.
16. dH_2O rinse.
17. Dehydrate through graded alcohols to xylene.
18. Cover slip with Permount.

Polyclonal Rabbit Anti-FoxP3 (Bioss, bs-0269R) Indirect Immunohistofluorescence on FFPE Mouse Tissue

1. Formalin-fixed paraffin-embedded tissue was cut at 5 µm, mounted on charged slides, then placed in an 80 °C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in three changes of xylene, five minutes each.
3. Hydrate slides through graded ethanols to deionized water.
4. Perform antigen retrieval in Tris-EDTA buffer, pH 9.0 (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20), for 3 minutes in a Biocare Decloaker (Biocare Medical, Concord, CA). Let slides cool to room temperature.
5. Serum block for 1 hour at room temperature using 10% goat serum in PBS.
6. Incubate with primary antibody diluted to 1:800 in PBS with 1% goat serum overnight at 4˚C.
7. Rinse for 5 minutes in PBS ×3.
8. Incubate with goat anti-rabbit Alexa Fluor 594 H&L (Life Technologies, Grand Island, NY) at 1:500 in PBS 30 minutes at room temperature in the dark.
9. Rinse for 5 minutes in PBS ×3.
10. Rinse in deionized water ×3.
11. Cover slip with ProLong Gold antifade reagent with DAPI (Life Technologies, Grand Island, NY).

Positive Control Tissue – Mouse Spleen

Mouse Anti-Galectin 3 mAb(Thermo Scientific, MA1-940, Clone A3A12)
Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue

1. Five µm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Slides were placed in an 80 °C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes ×3) and hydrated through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS] for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum at 1:200 overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent(HRP, Mouse)(Cell Signaling Technology, Beverly,MA) for 30 minutes at room temperature.
13. PBS rinse ×3.
14. Stain with DAB (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. dH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with tap H_2O for10 minutes.
18. dH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Rabbit anti-Phospho-Histone H2A.X Indirect Immunofluorescence Immunohistochemistry on Formalin Fixed Paraffin Sections

1. Deparaffinize and hydrate sections.
2. Rinse for 5 minutes in dH_2O.
3. Serum block for one hour at room temperature using 10% goat serum in PBS.
4. Rinse with PBS.
5. Incubate with primary antibody diluted 1:480 in PBS with 1% goat serum overnight at 4ºC.
6. Rinse for 5 minutes in PBS ×3.
7. Incubate with goat anti-rabbit Alexa Fluor 488 at 1:400 in PBS 30 minutes at room temperature in the dark.
8. Rinse for 5 minutes in PBS ×3.
9. Cover slip with Crystal Mount with propidium iodide at 1 µl/ml.

Note: Keep IF stained slides in the dark, at 4ºC, to prevent fading of the fluorphore!

Mouse Monoclonal Anti-Human Anti-Mitochondria Antibody, surface of intact mitochondria, clone 113-1 (Millipore, MAB1273) Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue

1. Five µm thick sections were cut from FFPE human tissue and mounted on charged slides.
2. Place slides in an 80°C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes ×3) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 9.0 Tris-EDTA solution (10 mM tris base, 1 mM EDTA, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at 1:1600 overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Mouse, Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
13. PBS rinse ×3.
14. Stain with DAB (Cell Signaling Technology, Beverly, MA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with tap H_2O for 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Anti-Human Nuclei Mouse Monoclonal (Chemicon, MAB4383) Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue

1. Five µm thick sections were cut from FFPE human tissue and mounted on charged slides.
2. Place slides in an 80 °C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes ×3) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at 1:800 overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Mouse, Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
13. PBS rinse ×3.
14. Stain with DAB (Cell Signaling Technology, Beverly, MA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with tap H_2O for 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Ki67 Mouse Monoclonal (VP-K452, Vector Laboratories, Inc.) Immunohistochemistry on Formalin Fixed Paraffin Embedded Tissue

1. Tissue was fixed for 24 hours in 10% neutral buffered formalin, dehydrated through graded ethyl alcohols, paraffin infiltrated, and paraffin embedded.
2. Tissue sections were cut at 5 μm and mounted on slides.
3. Slides were deparaffinized in xylene and hydrated through graded ethyl alcohols to water.
4. Antigen retrieval was performed in a pH 6.0 citrate buffer (10mM citric acid, 0.05% Tween 20) for 3 minutes in a Biocare decloaker (Biocare, Concord, CA).
5. Slides were washed with PBS three times.
6. Nonspecific binding was blocked with 10% goat serum in PBS for one hour and endogenous peroxidase was blocked with 0.3% hydrogen peroxide in PBS for 10 minutes.
7. The slides were then incubated with primary antibody (Ki67 Mouse Monoclonal, VP-K452, Vector Laboratories, Inc.) at a 1:800 dilution in PBS with 1% goat serum overnight at 4 °C.
8. After washing with PBS three times, the sections were incubated with Signal Stain® Boost IHC Detection Reagent (HRP, Mouse, Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
9. Following three washes in PBS, slides were developed with Incubate with Signal Stain® DAB Substrate Kit (Cell Signaling Technology, Beverly, MA), counterstained with Mayer's hematoxylin, dehydrated and cover slipped with Permount.

All reagents and chemicals are from Sigma, St. Louis, MO unless otherwise noted.

Anti-Melan A Rabbit Polyclonal (Abcam, ab118440) Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue

1. Five µm thick sections were cut from FFPE human tissue and mounted on charged slides.
2. Place slides in an 80° C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes ×3) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum at 1:800 overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit) (Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
13. PBS rinse ×3.
14. Stain with DAB (Cell Signaling Technology, Beverly, MA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with H_2O 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Rabbit Anti-human mTOR (Cell Signaling, #2972) Immunohistochemistry on FFPE Human Tissue

1. Slides were cut at 5 μm, mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80˚C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH_2O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature using 10% goat serum in PBS.
9. Incubate with primary antibody at 1:100 in PBS with 1% goat serum overnight at 4ºC.
10. Rinse for 5 minutes in PBS ×3.
11. Incubate with Signal Stain® Boost IHC Reagent(HRP, Rabbit, Cell Signaling #8114S) 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS ×3.
13. Stain with DAB(Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH_2O.
15. Counterstain 1 minute with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H_2O for 10 minutes.
17. Rinse in dH_2O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

Anti-NFkB2 Rabbit anti-mouse Polyclonal (Lifespan Biosciences, Inc., LS-B11757) Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue

1. Five µm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Place slides in an 80̊C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes ×3) and hydrate through graded alcohols to deionized water.
4. Perform HIER(heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at 1:400 overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly,MA) 30 minutes at room temperature.
13. PBS rinse ×3.
14. DAB substrate (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. dH_2O rinse.
16. Mayer's hematoxylin (Sigma, St. Louis, MO) 1 minute.
17. Rinse with tap H2O for 10 minutes.
18. dH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Anti-Nitrotyrosine (abcam, ab42789) Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue

1. Five μm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Slides were placed in an 80° C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes ×3) and hydrated through graded alcohols to deionized water.
4. HIER(heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
8. Serum block [10% goat serum (Sigma, St. Louis, MO) in PBS] 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.01% Triton X-100(Sigma, St. Louis, MO) at 1:50 overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly,MA) for 30 minutes at room temperature.
13. PBS rinse ×3.
14. DAB substrate (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. dH_2O rinse.
16. Mayer's hematoxylin (Sigma, St. Louis, MO) 1 minute.
17. Rinse with tap H_2O for 10 minutes.
18. dH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Positive control tissue – mouse liver

Rabbit Anti- PD-L1 (Cell Signaling, #13684) Immunohistochemistry on FFPE Human Tissue

1. Slides were cut at 5 μm, mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80 °C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH_2O.
5. Perform antigen retrieval in Tris-EDTA buffer pH 9.0 (10 mM Tris Base, 1 mM EDTA, 0.05% Tween 20) for three minutes in Biocare Decloaker chamber.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature. Use 10% goat serum in PBS.
9. Incubate with primary antibody at 1:100 in PBS with 1% goat serum overnight at 4ºC.
10. Rinse for 5 minutes in PBS ×3.
11. Incubate with Signal Stain® Boost IHC Reagent (HRP, Rabbit, Cell Signaling #8114S) for 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS ×3.
13. Stain with DAB (Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH_2O.
15. Counterstain for 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H_2O for 10 minutes.
17. Rinse in dH_2O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

Anti-Phospho-CREB (Cell Signaling, #9198 Rabbit mAb(Ser133) (87G3))
Immunohistochemistry on FFPE Mouse Tissue

1. Slides were cut at 5 μm, mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80˚C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH_2O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20), three minutes in Biocare Decloaker chamber.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature using 10% goat serum in PBS.
9. Incubate with primary antibody at 1:800 in PBS with 1% goat serum overnight at 4ºC.
10. Rinse for 5 minutes in PBS ×3.
11. Incubate with Signal Stain® Boost IHC Reagent (HRP, Rabbit, Cell Signaling #8114S) 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS ×3.
13. Stain with DAB (Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH_2O.
15. Counterstain for 1 minute with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H_2O for 10 minutes.
17. Rinse in dH_2O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

Rabbit Anti-mouse Phospho-Stat 3(Tyr705)(D3A7) (Cell Signaling, #9145 XP Rabbit mAb)) Immunohistochemistry on FFPE Canine Tissue

1. Slides were cut at 5 µm, mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80˚C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH_2O.
5. Perform antigen retrieval in Tris-EDTA buffer pH 9.0 (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20), three minutes in Biocare Decloaker chamber.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature using 10% goat serum in PBS.
9. Incubate with primary antibody at 1:400 in PBS with 1% goat serum and 0.1% Triton X-100 overnight at 4ºC.
10. Rinse for 5 minutes in PBS ×3.
11. Incubate with Signal Stain® Boost IHC Reagent (HRP, Rabbit, Cell Signaling #8114S) for 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS ×3.
13. Stain with DAB (Cell Signaling, #8059S) 1 minute at room temperature.
14. Rinse in dH_2O.
15. Counterstain for 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H_2O for 10 minutes.
17. Rinse in dH_2O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

Anti-Phospho-Akt (Ser473)(D9E) Rabbit Monoclonal (Cell Signaling, 4060S) Immunofluorescence on Formalin Fixed Paraffin Embedded Mouse Tissue

1. Five µm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Place slides in an 80 °C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes ×3) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Circle sections with Liquid Blocker Pap Pen (Newcomer Supply).
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS, Sigma, G6767 for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum at 1:100 overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Alexa Fluor Goat anti-rabbit 594 (H+L) for 30 minutes at room temperature.
13. PBS rinse ×3.
14. dH_2O rinse.
15. Cover slip with Prolong Gold Antifade Reagent with DAPI, (Life Technologies, P36931)

Anti-Phospho-Akt (Ser473)(D9E) Rabbit Monoclonal (Cell Signaling, 4060S) Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue

1. Five µm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Slides were placed in an 80° C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes ×3) and hydrated through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
8. Serum block with10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum at 1:100 overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly,MA) for 30 minutes at room temperature.
13. PBS rinse ×3.
14. Stain with DAB substrate (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. dH_2O rinse.
16. Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with tap H2O for 10 minutes.
18. dH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Rabbit Monoclonal Anti-human Phospho-mTOR(Ser2448)(49F9) (Cell Signaling, #2976) Immunohistochemistry on FFPE Human Tissue

1. Slides were cut at 5 μm, mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80˚C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH_2O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20), three minutes in Biocare Decloaker.
6. Cool slides for 30 minutes
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature. Use 10% goat serum in PBS.
9. Incubate with primary antibody at 1:100 in PBS with 1% goat serum overnight at 4ºC.
10. Rinse for 5 minutes in PBS ×3.
11. Incubate with Signal Stain® Boost IHC Reagent (HRP, Rabbit, Cell Signaling #8114S) for 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS ×3.
13. Stain with DAB (Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH_2O.
15. Counterstain for 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H_2O for 10 minutes.
17. Rinse in dH_2O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

Rabbit Anti-Phospho-S6 Ribosomal Protein (Ser235/236) (Cell Signaling, #2211) Immunohistochemistry on FFPE Human Tissue

1. Slides were cut at 5 μm, mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80˚C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH_2O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20), three minutes in Biocare Decloaker chamber.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature. Use 10% goat serum in PBS.
9. Incubate with primary antibody at 1:100 in PBS with 1% goat serum overnight at 4ºC.
10. Rinse for 5 minutes in PBS ×3.
11. Incubate with Signal Stain® Boost IHC Reagent (HRP, Rabbit, Cell Signaling #8114S) for 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS ×3.
13. DAB (Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH_2O.
15. Counterstain for 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H_2O for 10 minutes.
17. Rinse in dH_2O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

Anti-PTEN (D4.3) Rabbit Monoclonal (Cell Signaling, 9188) Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue

1. Five µm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Slides were placed in an 80° C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes ×3) and hydrated through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 20 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at 1:100 overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly,MA) for 30 minutes at room temperature.
13. PBS rinse ×3.
14. Stain with DAB (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. dH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with tap H_2O for 10 minutes.
18. dH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Polyclonal Rabbit Anti-SDF1 (Bioss, bs-4938R) Indirect Immunohistofluorescence on FFPE Mouse Tissue

1. Formalin-fixed paraffin-embedded tissue was cut at 5 µm, mounted on charged slides, then placed in an 80˚C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in three changes of xylene, five minutes each.
3. Hydrate slides through graded ethanols to deionized water.
4. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mMcitric acid, 0.05% Tween 20), for 3 minutes in a Biocare Decloaker (Biocare Medical, Concord, CA). Let slides cool to room temperature.
5. Serum block for 1 hour at room temperature. Use 10% goat serum in PBS.
6. Incubate with primary antibody diluted to 1:200 in PBS with 1% goat serum overnight at 4˚C.
7. Rinse for 5 minutes in PBS X 3.
8. Goat anti-rabbit Alexa Fluor 594 (H&L, Life Technologies, Grand Island, NY) at 1:500 in PBS 30 minutes at room temperature in the dark.
9. Rinse for 5 minutes in PBS X 3.
10. Rinse in deionized water X 3.
11. Cover slip with ProLong Gold antifade reagent with DAPI (Life Technologies, Grand Island, NY).

Positive Control Tissue – Mouse Spleen

Mouse Anti-Stat 3(124H6) (Cell Signaling, #9139 Mouse mAb)) Immunohistochemistry on FFPE Canine Tissue

1. Slides were cut at 5 µm, mounted on charged slides, and dried at room temperature overnight.
2. Place slides in 80˚C oven for 20 minutes to melt paraffin.
3. Deparaffinize in 3 changes of xylenes and hydrate sections through graded alcohols to water.
4. Rinse for 5 minutes in dH_2O.
5. Perform antigen retrieval in citrate buffer pH 6.0 (10 mM citric acid, 0.05% Tween 20) for three minutes in Biocare Decloaker chamber.
6. Cool slides for 30 minutes.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes.
8. Serum block for 60 minutes at room temperature. Use 10% goat serum in PBS.
9. Incubate with primary antibody at 1:600 in PBS with 1% goat serum and 0.1% Triton X-100 overnight at 4ºC.
10. Rinse for 5 minutes in PBS ×3.
11. Incubate with Signal Stain® Boost IHC Reagent(HRP, Mouse) (Cell Signaling #8125S) 30 minutes at room temperature.
12. Rinse for 5 minutes in PBS ×3.
13. Stain with DAB (Cell Signaling, #8059S) for 1 minute at room temperature.
14. Rinse in dH_2O.
15. Counterstain 1 min with Mayer's hematoxylin (Sigma, MHS32).
16. Rinse in tap H_2O for 10 minutes.
17. Rinse in dH_2O for 5 minutes.
18. Dehydrate through graded alcohols to xylene.
19. Coverslip with Permount.

TUNEL Immunohistochemistry on FFPE Sections

(*In situ* Cell Death Detection Kit,POD, #11684817910, Roche Diagnostics, Pengberg, Germany)

1. Sections were cut at 5 µm and mounted on slides. Slides were placed in oven at 80˚C for 20 minutes to melt paraffin. Slides were then deparaffinized in three changes of xylenes, five minutes each, then hydrated through graded ethanols to deionized water.
2. Wash in PBS, pH 7.4, for 30 minutes at room temperature.
3. Block endogenous peroxidase with 3% H_2O_2 in PBS for 10 minutes at room temperature.
4. Wash in PBS for 5 minutes.
5. Incubate in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 8 minutes.
6. Incubate positive control sections in 30 U/ml DNase grade 1 in PBS 10 minutes at room temperature (50µl/slide).
7. Prepare TUNEL reaction mixture and keep on ice:
    a. Remove 100 µl Label Solution (vial 2) for negative controls.
    b. Add total volume (50 µl) of Enzyme Solution to the remaining 450 µl label solution in vial 2 for 500 µl TUNEL reaction mixture.
    c. Mix well.
8. PBS 5 minutes ×2.
9. Add 50 µl TUNEL reaction mixture to samples:
    a. Use 50 µl per slide Label Solution for negative controls.
    b. Incubate for 1 hour at 37 °C in the dark.
10. Wash in PBS for 5 minutes ×3.
11. Incubate with 50 µl/slide Converter-POD(vial 3) for 30 minutes at 37 °C.
12. Wash in PBS for 5 minutes ×3.
13. Stain with DAB until developed (DAB substrate kit for peroxidase, Vector Laboratories, Inc., Burlingame, CA)
14. Wash in dH_2O for 10 minutes.
15. Counterstain with Mayer's hematoxylin for 30 seconds.
16. Tap water rinse for 10 minutes.
17. Wash in dH_2O 10 minutes.
18. Cover slip with Permount.

Anti-VEGF Receptor 2(55B11) Rabbit Monoclonal (Cell Signaling, #2479)
Immunohistochemistry on Formalin Fixed Paraffin Embedded Mouse Tissue

1. Five µm thick sections were cut from FFPE murine tissue and mounted on charged slides.
2. Place slides in an 80̊C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes ×3) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 9.0 EDTA-Tris solution (10mM Tris Base, 1 mM EDTA, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS fpr 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum at 1:1200 overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit) (Cell Signaling Technology, Beverly, MA) for 30 minutes at room temperature.
13. PBS rinse ×3.
14. Stain with DAB (Cell Signaling Technology, Beverly, MA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) 1 minute.
17. Rinse with tap H2O for 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Mouse Anti-Vimentin (Dako, M0725, clone V9) and Monoclonal Rabbit Anti-E-cadherin(24E10) (Cell Signaling, 3195S) Indirect Immunohistofluorescence Costaining on FFPE Human Tissue

1. Formalin-fixed paraffin-embedded tissue was cut at 5 µm, mounted on charged slides, then placed in an 80˚C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in three changes of xylene, five minutes each.
3. Hydrate slides through graded ethanols to deionized water.
4. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM Citric acid, 1 mM EDTA, 0.05% Tween 20), for 3 minutes in a Biocare Decloaker (Biocare Medical, Concord, CA). Let slides cool to room temperature.
5. Serum block for 1 hour at room temperature using 10% goat serum in PBS.
6. Incubate with anti-vimentin antibody diluted to 1:6400 and anti-Ecadherin diluted to 1:200 in PBS with 1% goat serum overnight at 4˚C.
7. Rinse for 5 minutes in PBS ×3.
8. Goat anti-mouse Alexa Fluor 555 (H+L) and Goat anti-rabbit Alexa Fluor 488 (H+L) at 1:500 in PBS 30 minutes at room temperature in the dark.
9. Rinse for 5 minutes in PBS ×3.
10. Rinse in deionized water ×3.
11. Cover slip with Crystal Mount (Biomeda, M03).

Mouse Anti-Vimentin (Dako, M0725, clone V9) Indirect Immunohistofluorescence on FFPE Human Tissue

1. Formalin-fixed paraffin-embedded tissue was cut at 5 µm, mounted on charged slides, then placed in an 80˚C oven for 20 minutes to melt the paraffin.
2. Deparaffinize in three changes of xylene, five minutes each.
3. Hydrate slides through graded ethanols to deionized water.
4. Perform antigen retrieval in citrate buffer, pH 6.0 (10 mM Citric acid, 1 mM EDTA, 0.05% Tween 20), for 3 minutes in a Biocare Decloaker chamber (Biocare Medical, Concord, CA). Let slides cool to room temperature.
5. Serum block for 1 hour at room temperature using 10% goat serum in PBS.
6. Incubate with primary antibody diluted to 1:6400 in PBS with 1% goat serum overnight at 4˚C.
7. Rinse for 5 minutes in PBS ×3.
8. Incubate with goat anti-mouse Alexa Fluor 555 (H&L) at 1:500 in PBS 30 minutes at room temperature in the dark.
9. Rinse for 5 minutes in PBS ×3.
10. Rinse in deionized water ×3.
11. Cover slip with Crystal Mount (Biomeda, M03).

Anti-Vimentin (D21H3) XP® Rabbit Monoclonal (Cell Signaling, 5741)
Immunohistochemistry on Formalin Fixed Paraffin Embedded Human Tissue

1. Five µm thick sections were cut from FFPE human tissue and mounted on charged slides.
2. Slides were placed in an 80° C oven for 20 minutes to melt the paraffin.
3. Slides were deparaffinized in xylene (5 minutes ×3) and hydrated through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 9.0 Tris-EDTA solution (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum at 1:400 overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly, MA) 30 minutes at room temperature.
13. PBS rinse ×3.
14. Stain with DAB (Cell Signaling Technology, Beverly, MA) for 1 minute.
15. DH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with tap H2O for 10 minutes.
18. DH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.

Anti-YY1 Rabbit Polyclonal (Santa Cruz, sc-1703) Immunohistochemistry on Formalin Fixed Paraffin Embedded Canine Tissue

1. Five µm thick sections were cut from FFPE mouse tissue and mounted on charged slides.
2. Place slides in an 80̊C oven for 20 minutes to melt the paraffin.
3. Deparaffinize in xylene (5 minutes ×3) and hydrate through graded alcohols to deionized water.
4. Perform HIER (heat induced epitope retrieval) in pH 6.0 citrate solution (10mM citric acid, 0.05% Tween 20) or pH 9.0 Tris-EDTA solution (10mM Tris base, 1 mM EDTA, 0.05% Tween 20) for 3 minutes in Decloaking Chamber (Biocare Medical, Concord, CA).
5. Let slides cool for 20 minutes.
6. PBS rinse.
7. Block endogenous peroxidase with 0.3% H_2O_2 in PBS for 10 minutes at room temperature.
8. Serum block with 10% goat serum (Sigma, St. Louis, MO) in PBS for 1 hour at room temperature.
9. PBS rinse.
10. Incubate with primary antibody in PBS with 1% goat serum and 0.1% Triton X-100 at overnight at 4 °C.
11. PBS rinse ×3.
12. Incubate with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Beverly,MA) for 30 minutes at room temperature.
13. PBS rinse ×3.
14. Stain with DAB (Vector Laboratories, Inc., Burlingame, CA) for 1 minute.
15. dH_2O rinse.
16. Counterstain with Mayer's hematoxylin (Sigma, St. Louis, MO) for 1 minute.
17. Rinse with tap H2O for 10 minutes.
18. dH_2O rinse.
19. Dehydrate through graded alcohols to xylene.
20. Cover slip with Permount.